A Step Closer to Understanding Immobilized Organo- and Bio-Molecular Complexes on Solid Surfaces

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Organic reactions on plane metal surfaces are of much interest for applications in biosensors, biomaterials, and biochips. However, the details of the reactions are not easy to characterize because surface-sensitive technologies cannot fully determine molecular structures, and collecting enough grafted materials for sufficient bulk analyses is tremendously difficult. In this study, a well-known amine-reactive cross-linking reaction for biomolecular conjugation occurs on an amine-pendant self-assembled monolayer on gold. The infrared reflection absorption spectroscopy (IRRAS) of that reaction and other analogous reactions produces significantly different spectra. With the help of x-ray photoelectron spectroscopy (XPS) and near-edge x-ray absorption fine structure spectroscopy (NEXAFS), we deduced that a so-called side reaction, accompanying the main reaction, occurred in this case. Furthermore, a peptide bearing both a free amino and a free thiol group was immobilized as two configurations on the surface, either one-end or two-end fixed.

Self-assembled monolayers (SAMs) on metal surfaces such as gold, silicon, and titanium have been the subject of intense investigations over the past 20 years. The driving force for these endeavors is the goal of combining micro- and nano-electronics with biotechnology. Unlike in a bulk reaction, however, where the pure product from organic reactions can be separated and definitely identified by well-established analysis methods such as nuclear magnetic resonance (NMR), elemental analysis, and x-ray single crystal diffraction, the molecular structure of a surface product on a plane metal surface can only be deduced from surface-sensitive analysis techniques, such as IRRAS, XPS, NEXAFS, and time-of-flight secondary ion mass spectroscopy (TOF-SIMS). But each of these methods provides partial information only. Therefore, an integrated analysis and logical interpretation of the results from these surface-sensitive techniques is necessary.

We investigated a well known cross-linking reaction on gold surfaces as a method for biomolecular immobilization, i.e. fixing biological molecules onto a substrate (**Scheme I**). The reaction includes three steps: 1) the activation of the inert gold surface via the self-assembly an amino-terminated species, cystamine, which contains both a disulfide and two end-amines; 2) the subsequent surface reaction of the primary amines with hetero-bifunctional cross-linkers bearing both an amine-reactive species, succinimidyl ester (NHS), and a thiol-reactive species, maleimide; and finally 3) the covalent attachment of biomolecules onto the surface.

In step 2, we found that a surface derived from N-succinimidyl-6-maleimidyl-hexanoate (SMH) presented a specific IRRAS spectrum, where four bands were observed in the carbonyl stretching region (1700-1900 cm^{-1}), while only one band around 1710 cm^{-1} was observed for its analogous surfaces (**Figure 1**). It is widely believed that the reaction occurs between amine and NHS, eliminating NHS as the leaving group and producing a maleimide-terminated surface. In this case, due to the asymmetric stretching mode of maleimide,

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the infrared spectra should exhibit one strong band around 1710 cm^{-1}. What caused the other three bands? We first thought that either a side reaction or an orientation of maleimide might account for them. Since NEXAFS studies for the orientation of functional groups are well understood by our colleagues, G. Hähner and D. Brovelli from the Laboratory of Surface Science and Technology at the Swiss Federal Institute of Technology in Switzerland, they collected the NEXAFS data at beamline U1A on our behalf. The results (**Figure 2**) confirmed the existence of the double bond (C=C) of maleimide and the random orientation of C=C and C=O. This measurement helped us to identify the real culprit, a side reaction of amine with maleimide, and thus the resulted terminal NHS groups, for the anomalous IRRAS spectrum. Further, we cross-proved our deduction with another experiment: the synthesis of the expected surface product, N,N'-bis (maleimidylhexanoyl)cystamine (BMHC), in which BMHC self-assembles on gold surfaces. The IRRAS spectrum of BMHC only presents one strong band around 1710 cm^{-1}, corresponding to the maleimide-terminated surfaces, but is different than the spectrum for the SMH-derived surface that contains NHS.

Finally, a cell-adhesive peptide bearing both a free amino and a free thiol group was grafted to two functionalized surfaces, a singular maleimidyl-pendant (BMHC-derived) surface and a two-linker (SMH-derived maleimide and NHS) modified surface, respectively. The first reaction produced an amino-terminated peptide structure via a thioether linkage; the latter produced a bridging peptide structure via both amide and thioether linkages. The above conclusions are carefully deduced from IRRAS measurements, and additionally supported by XPS, NEXAFS, and TOF-SIMS.

The different configurations of biomolecules on solid surfaces will have a significant impact on the development of applications in biomaterials, biosensors, and biochips. The geometry and topology of the attached biomolecules affect surface charges, hydrophobic and hydrophilic properties, molecular orientations, and native conformations. In turn, these surface properties determine the molecules' biological activity and function.

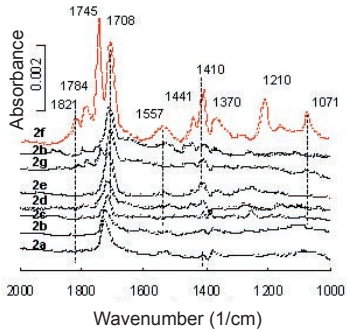


Figure 1. IRRAS spectra of a series of linker-functionalized surfaces, where the side-reaction-resulted curve 2f (surface 2 in Scheme 1) on the top is significantly different from its analogs (2a-g, 2h).

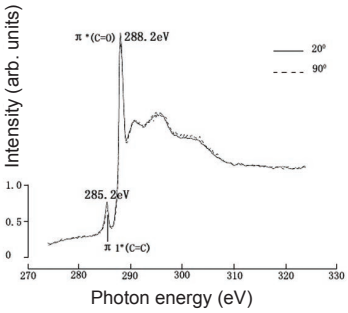
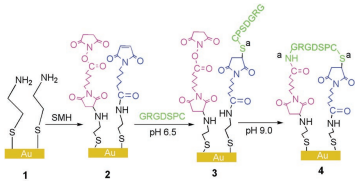


Figure 2. NEXAFS spectra of surface 2 in Scheme 1 at the C 1s edge recorded for two angles of incidence. The overlapping of two lines between grazing (20°) and normal (90°) angles indicates the random orientation of maleimidyl groups.



Scheme 1. Sketch of the subsequent surface reactions. 1) cystamine SAMs, 2) a side-reaction-resulted two-linker (maleimide and NHS)-terminated surface from SMH, 3) a one-end fixed peptide, and 4) a two-end fixed peptide. The linkage atoms S and N (marked *a)* are highlighted from amino acids Cys and Gly, respectively, but the peptide sequence H-Gly-Arg-Gly-Asp-Ser-Pro-Cys-OH (GRGDSPC) is still kept. SMH is N-succinimidyl-6-maleimidylhexanoate.